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## Description

The present invention relates to a method for the determination of a multivalent immune species, such as Streptococcus A, present in a biological sample. It also relates to a test kit useful in carrying out this method.

The antigen-antibody reaction is the basis for all immunological test methods. Certain proteins known as antibodies are produced by mammals in response to the presence of an antigen, that is a foreign substance, which can be another protein or a carbohydrate. This normal body response to a foreign substance has led to the development of a number of techniques which are used to diagnose various diseases, disorders and physiological conditions. In a general sense, the component of the antibody-antigen reaction to be detected is defined herein as the immune species while the other corresponding component is considered the receptor.

In vitro tests for the presence of a suspected protein, antigen or antibody in a biological sample are carried out by adding the immunological counterpart to the biological sample. If the suspected substance is present, the resulting antigen-antibody reaction can be demonstrated by precipitation of the antigen-antibody complex. This reaction complex is generally difficult to detect visually. For this reason, either antibodies or antigens are often bound to insoluble particles, for example polymer latex particles, so that when the complex is formed, it is readily detectable from the resulting agglutination either by observing the presence of clumping or a detectable tracer associated with the particles. Agglutination then is characterized by the clumping of particles from a suspension of particles. Further details of known agglutination methods are provided in U.S. Patents 4,419,453 and 4,459,361.

Of the several groups of Streptococci, group A streptococcus (*S. pyogenes*) is primarily responsible for causing pathological conditions in humans, such as B-hemolytic pneumonia, scarlet fever, rheumatic fever, cardiac sequelae, glomerulonephritis, septic sore throat and puerperal sepsis. Because of the serious nature of infections potentially caused by Streptococcus A, it is important to diagnose its presence in an early stage of infection so that an appropriate course of treatment may be selected. Early tests for detection required culturing a biological sample for long periods of time, usually at least 18 and up to 48 hours. In most cases, such lengthy tests delay treatment making them undesirable.

More recent tests for Streptococcus A have been described which are allegedly quicker than the culturing techniques. U.S. Patent 4,618,576 describes an agglutination test using certain enzymes to extract the antigen directly from the swab used to obtain a specimen from the throat. A kit comprising an applicator means for collecting the specimen, an extraction reagent containing the enzymes and suitable indicator reagents is also described. The described method comprises placing the extracted antigen in the wells of a sample plate along with latex particles coated with antibodies. After agitating the wells mechanically to facilitate antigen-antibody reaction, any agglutination is observed in the mixture in the wells. This method is disadvantageous because the agglutinate is not readily observable except with a microscope, and requires extraction enzymes prepared from a bacterium which must be cultured.

Other agglutination assays using various latex particles and coloring techniques for observing the agglutinate are described in E.P. Publications 150,567 and 174,195. U.S. Patent 4,552,839 describes an agglutination assay carried out by concentrating particles in a small area on a solid surface. The concentrated particles can have antibodies or label attached thereto to aid in detection of the analyte, that is, the antigen. The conditions of the assay are chosen to adjust the reaction of beads to each other. For example, the ionic strength of the aqueous medium is adjusted based on the natural charge of the particles in order to maximize agglutination. Generally, the ionic strength is varied from 0.0001 to 0.1. Once the agglutinate is formed and concentration on a bibulous material (for example, filter paper) is effected, a phosphate buffered saline wash containing a surfactant is applied to aid in wicking and separating unbound materials from bound materials (Col. 6, lines 50-55).

Japanese Patent Publication 57-35754 describes an agglutination assay wherein a salt is included in the assay medium to prevent non-specific agglutination during the assay.

Current agglutination assays for a number of multivalent immune species [for example, Streptococcus A, human chorionic gonadotropin (hCG), Chlamydia, Gonorrhea, herpes, HIV and HTLV viruses and others] are limited in usefulness in several respects. They are generally difficult to interpret, non-quantitative, subject to interferences and often insensitive. In order to improve the sensitivity and accuracy of agglutination assays for multivalent immune species carried out using antibody-bound particles, it has been found that a means is needed for keeping agglutinated materials from breaking apart and, at the same time, effectively separating agglutinated and unagglutinated materials.

The problems observed with known agglutination assays have been solved with an agglutination method for the determination of a multivalent immune species comprising:

(a) contacting an aqueous liquid containing the species in free form with a reagent comprising water-insoluble particles having tracer molecules associated therewith and receptor molecules reactive with the species bound to the surface thereof, so as to form an agglutinate of the reaction product of the species and the receptor molecules,

5 (b) simultaneously or subsequent to contacting step (a), contacting the agglutinate with a microporous water-insoluble membrane having an average pore size which is at least 5 times greater than the average diameter of the water-insoluble particles,

(c) washing unagglutinated residual materials through the membrane while leaving the agglutinate thereon with a wash solution, and

10 (d) determining the amount of tracer either in the agglutinate remaining on the membrane or the residual materials,

the method characterized wherein the wash solution has a pH of from 5 to 10 and an ionic strength of at least 0.25.

In addition, the present invention provides a test kit for the determination of a multivalent immune species comprising:

15 an agglutination indicator reagent comprising water-insoluble particles having tracer molecules associated therewith and receptor molecules reactive with the species bound to the surface thereof, and

a wash solution,

the kit characterized wherein the wash solution has a pH of from 5 to 10 and an ionic strength of at least 0.25.

20 The present invention provides an agglutination assay which is highly accurate, easy to read, rapid and sensitive to the presence of the multivalent immune species, such as Streptococcus A, in an aqueous liquid such as a biological sample. This assay is quantitative, not merely qualitative. These advantages are achieved by carrying out the assay on a microporous membrane which has an average pore size which is at least five times greater than the average diameter of the water-insoluble particles carrying the species receptor. The particles readily agglutinate when the receptor and species react. It was found unexpectedly that the agglutinate remains on the membrane despite the relatively large pore size because a particular wash solution is used for washing unagglutinated residual materials from the agglutinate residing on a microporous membrane. The wash solution has a pH of from 5 to 10 and an ionic strength of at least 0.25, as described in more detail below. Because of the relatively high ionic strength of the wash solution, the agglutinate holds together very well when unagglutinated residual materials are washed away. The result is effective separation and a more accurate, quantitative assay.

The present invention provides a diagnostic test for a multivalent immune species which can be performed in a very short time, that is less than 10 minutes, and without the use of complicated equipment. This permits the test to be performed in a doctor's office and enables the doctor to determine a course of treatment based upon the results of the test the same day. The test detects the presence of the species, such as Streptococcus A antigen, Chlamydia or Gonorrhea antigen, or hCG in a biological sample, such as a swab specimen from the throat, urine specimen or sample of another aqueous liquid. Such biological samples can be tested with or without pretreatment (for example, filtration) to remove unwanted debris or interferents.

40 In accordance with this invention, a test kit provides the materials and reagents needed for carrying out the method of the invention. This kit generally includes (1) an agglutination indicator reagent comprising receptor molecules for the multivalent immune species bound to water-insoluble particles having tracer molecules associated therewith and (2) a wash solution having the properties noted herein. Optionally and preferably, the kit also includes a composition for extracting the species from a biological specimen if the species is not already in free form. Also optionally, the kit can comprise a neutralizing solution to neutralize the extraction composition after extraction has occurred or a suitable applicator means for collecting the biological specimen. An applicator means usually includes an applicator stick and a fibrous swab at one end thereof. Useful applicator means for Streptococcus A tests are known in the art and include those described, for example, in U.S. Patent 4,618,576. All of these kit components are described in more detail below.

45 The method of this invention can be used to detect and quantify any of a wide variety of multivalent immune species. Such species are generally antigens which have at least two sites for complexing with the corresponding receptor, that is, corresponding antibodies. Alternatively, the multivalent species to be detected can be an antibody which has at least two complexing sites reactive with the corresponding antigen or an anti-antibody. Multivalent immune species which can be detected with this invention include, but are not limited to, Streptococcus A antigen, antigens from chlamydial and gonococcal organisms, HIV or HTLV antigens or antibodies, human chorionic gonadotropin (hCG), luteinizing hormone (LH), herpes

viruses, drugs, antibiotics, and other hormonal, bacterial or viral antigens and antibodies. The species can be detected in free form which means that it is readily accessible to reactive receptor molecules. In some instances, the species must be extracted from the organisms or virus found in the biological specimen. In other instances, the species is already in free form and requires no extraction procedures prior to the assay.

5 Extraction procedures for a given species are known to one skilled in the art. Exemplary extraction procedures for Streptococcus A antigen are described below.

Preferably, the invention is used to detect Streptococcus A antigen as is demonstrated in the following embodiment and in Example 1 below. This embodiment of the invention relating to Streptococcus A antigen is presented for illustrative purposes, but it will be understood that the scope of the invention is not so  
10 limited. A biological sample suspected of containing the antigen can be collected from a patient in any suitable manner. However, generally an applicator means is used to collect a biological sample by contacting the area of suspected infection with the applicator swab thereby collecting cells of Streptococcus A organisms if they are present. Subsequently, the antigens are extracted from the organisms in a suitable manner. A preferred extraction procedure includes dipping the swab in a suitable extraction composition  
15 containing one or more reagents which singly or in combination cause release of the Streptococcus A antigen from the organism, specimen cells and other debris in the sample.

Useful extraction compositions known in the art include a mixture of nitrite salt and glacial acetic acid, as described in E.P. Publication 150,567, noted above. Another extraction procedure involves the use of enzymes derived from the bacterium Streptomyces albus as described in U.S. Patent 4,618,576. A  
20 preferred extraction composition is a mixture of a nitrite salt (for example, sodium nitrite or potassium nitrite) with a nonvolatile organic acid (for example, succinic acid, malonic acid, citric acid and others known in the art). When used in the method of this invention, these two reagents of the composition combine to form a solution of nitrous acid which is a strong extraction reagent.

Extraction can be accompanied by incubation for a short period of time if desired. Centrifugation can  
25 also be used to remove extraneous material. After extraction, the medium containing the extracted antigen can be neutralized if necessary to bring the medium pH to that appropriate for antigen-antibody reaction. For example, when extraction is carried out with citric acid and a nitrite salt, the pH is lowered below that optimally desired for reaction. In that case, the medium is neutralized with a suitable buffer. Such optional steps are noted, for example, by Slifkin and others, J. Clin. Microbiol. 15(1), pp. 187-189, 1982.

30 The presence of a multivalent immune species in free form, for example, Streptococcus A antigen, is detected by an agglutination indicator reagent which comprises water-insoluble particles having tracer molecules associated therewith and receptor molecules (for example, antibodies to Streptococcus A antigen) reactive with the species bound in a suitable manner to the surface of the particles. Reaction (or binding) between immune species and receptor then results in a linking together of the particles so that  
35 they agglutinate and precipitate out of suspension.

Suitable particles useful in the indicator reagent can be natural or synthetic particles which are water-insoluble and capable of having a suitable number of tracer molecules associated therewith in some manner. Examples of useful particles include ferritin crystals, agarose particles, glass beads, polymeric particles, such as latex particles, and others known in the art. The following references describe  
40 representative useful particles: U.S. Patents 3,700,609, 3,853,987, 4,108,972, 4,401,765, 4,419,453, 4,459,361, 4,478,946 and 4,591,571. The particles useful in this invention are generally quite small, that is less than 1 micrometer in diameter. Preferably, they have an average diameter of from 0.1 to 0.7, and most preferably from 0.3 to 0.5, micrometer.

Particularly useful particles are polymeric latex particles, and more preferably they are what are known  
45 in the art as core-shell polymeric latex particles. A wide variety of monomers can be used in the preparation of such particles as long as the particles are water-insoluble. A worker skilled in the polymer chemistry art would be able to design and prepare suitable latex particles. Preferred core-shell polymeric latex particles in the practice of this invention are described in the examples below. These particles have a core composed of homo- or copolymers of styrene, and a shell composed of homo- or copolymers of chloromethylstyrene.

50 The particles useful in the practice of this invention have sufficient tracer molecules associated therewith in order to allow quantitative determination of the species from the amount of tracer seen in either the agglutinate or in the unagglutinated residual materials. The tracer molecules can be suitably attached to the other surface of the particles, or preferably, distributed within the particles. Any tracer material which allows detection of the agglutinate can be used. If ferritin crystals are used as the particles, the tracer  
55 molecules are molecules of iron inherently in those crystals. Other natural or synthetic particles can have, as tracers: radioisotopes, colorimetric dyes, fluorescent compounds, chemiluminescent compounds, phosphorescent compounds and other detectable materials known in the art. Preferably, the tracer is a radioisotope, colorimetric dye or fluorescent compound (for example, dye or rare earth chelate). A worker

skilled in the art would be able to combine an appropriate tracer with the particular particle used.

In one embodiment, the tracer can be a fluorescent rare earth chelate such as a europium chelate, as described for example, in U.S. Patent 4,259,313. In another and preferred embodiment, the tracer is a colorimetric dye which is readily detected in the agglutinate. Useful dyes are known in the art. Some dyes  
5 can be incorporated into the particles when the particles are prepared. Alternatively, the dyes are imbibed into preformed particles in such a manner that they do not leach out.

The tracer can be distributed within the particles in any suitable manner. For example, the tracer can be uniformly distributed therein as shown for example in U.S. Patent 3,853,987. Preferably, the tracer molecules are located in a restricted area of the particles, for example, near the surface or predominantly in  
10 the interior thereof. In the preferred core-shell particles, the tracer can be in either the core or shell, but most preferably, it is substantially in the core of the particles. In other words, very little (for example, less than 5% by weight) of the dye is in the shell portion of the particles.

Receptor molecules (for example, antibodies) reactive to the immune species to be detected, such as Streptococcus A antigen, are bound to the outer surfaces of the particles in a suitable manner, for example  
15 by adsorption or covalent attachment. Attachment can be achieved using known techniques, as described for example in the references cited above. Covalent attachment is preferred as the receptor molecules are less likely to be removed from the particles. When covalently attached, the receptor molecules can be bound directly to the particles or through suitable linking groups. When the receptor molecules are antibodies, either monoclonal or polyclonal antibodies can be used, but monoclonal antibodies are  
20 preferred. Antibodies can be obtained commercially or prepared using known techniques. Polyclonals, for example, are generally prepared by injecting antigen into suitable mammals which then generate the antibodies which can be removed for use. Monoclonals are obtained using conventional hybridoma technology. When the receptor molecules are antigens, the desired antigen molecules can be obtained using known procedures for isolating the molecules from suitable biological sources. For example, HIV or  
25 HTLV antigens can be obtained from the sera of patients infected with the virus.

Simultaneously or subsequent to contact of extracted multivalent immune species with receptor molecules to form the agglutinate, the agglutinate is also contacted with a microporous water-insoluble membrane. In one embodiment, the agglutinate can be formed in a separate container and then brought into contact with the membrane. Alternatively and preferably, the agglutinate is formed in the presence of  
30 the membrane. This membrane (described in detail below) can be simply a filter means held by hand through which unagglutinated materials are filtered. Preferably, however, it is mounted in a test device in which the assay is carried out. Such a test device is also described below.

Any microporous water-insoluble membrane can be used as long as it is inert to the materials used in the assay, and has the desired porosity which will allow fluids and nonagglutinated materials to pass through but which will retain agglutinated materials. In other words, the membrane pores must be large enough to allow passage of the indicator reagent, antigen and unagglutinated particles, but not large enough to allow agglutinated particles to pass through. More particularly, the average pore size of the membrane must be at least five times the average diameter of the water-insoluble particles described above. Preferably, the average pore size is from 6 to 15 times the average particle diameter. Useful membranes  
40 include polymeric materials which are commercially available from various sources. One useful membrane is a nylon 66 microporous membrane manufactured and marketed by Pali Corporation as BIODYNE or ULTIPOR.

A suitable incubation period can be used to optimize agglutination, if desired, before or during contact with the membrane. After that period, unagglutinated residual materials are washed through the membrane  
45 while leaving the agglutinate thereon. A critical feature of this invention is carrying out this wash step with a wash solution which has a pH of from 5 to 10 and which contains an ionic compound as described below. Preferably, the solution is buffered to a pH of from 6 to 9.

Any suitable organic or inorganic buffer can be used in the wash solution as long as it provides the desired pH. Useful buffers include glycine, tricine, 2-(N-morpholino)ethanesulfonic acid, 3-(N-morpholino)-  
50 propanesulfonic acid and others known to one skilled in the art.

The wash solution contains one or more ionic compounds which are present at a concentration sufficient to give the solution an ionic strength of at least 0.25. Preferably, the solution ionic strength is from 0.5 to 3. Ionic compounds can be used to supply the desired ionic strength. Such compounds are those which are ionized in aqueous solution to a high degree. Such compounds can be monovalent salts such as  
55 sodium chloride, potassium chloride and others known to one skilled in the art. Sodium chloride is preferred. Alternatively, the ionic compound can be a compound which is sufficiently ionized at the pH of the wash solution, but which may not be completely ionized under all conditions. Examples of such compounds include buffers, such as tricine, glycine, sodium glycinate, sodium tricine, 3-(N-morpholino)-

propanesulfonic acid, sodium salt and others apparent to one skilled in the art.

Once the unagglutinated residual materials have been washed through the membrane, the amount of multivalent immune species in either the agglutinate or residual materials can generally be determined with the unaided eye if the tracer is a readily viewable colorimetric dye. Otherwise, standard colorimetric detection equipment can be used. Other types of tracers, for example, radioisotopes, fluorescent dyes, phosphorescent dyes, and the like, require suitable detection equipment.

In a preferred embodiment, a method for detecting *Streptococcus A* comprises:

- (i) providing an applicator including an applicator stick and a fibrous swab and collecting a biological sample with the swab,
- (ii) providing an extraction composition comprising sodium nitrite and citric acid for effecting release of *Streptococcus A* antigen from the swab, dipping the swab in the extraction composition and incubating the swab within the extraction composition for a period of time sufficient to release antigen from the swab,
- (iii) neutralizing the solution of extracted antigen,
- (iv) contacting the neutralizing solution of extracted antigen with an agglutination indicator reagent comprising water-insoluble, polymeric core-shell latex particles containing tracer molecules substantially in the particle cores and comprising antibodies reactive with the antigen bound to the surface thereof, so as to form an agglutinate of the reaction product of the antigen and the antibodies, the contacting being carried out in the presence of a microporous water-insoluble membrane mounted in a disposable test device, the membrane having an average pore size which is at least five times the average diameter of the water-insoluble particles described above,
- (v) washing unagglutinated residual materials through the membrane while leaving the agglutinate thereon with a wash solution as described above, and
- (vi) determining the amount of tracer in the agglutinate remaining on the membrane.

While the present invention is not so limited, the assay for a multivalent immune species can be carried out using a suitable test device which comprises the microporous membrane described herein. Such a device can have one or more sample wells into which extracted antigen is deposited for reaction with the agglutination indicator reagent. This reagent can be added to the device during the assay, or incorporated therein at the time of manufacture. Once the agglutinate is formed, the unagglutinated residual materials can be washed through the membrane with the wash solution into a separate compartment below the membrane.

#### Example 1: Comparative Example for the Determination of *Streptococcus A*

This example demonstrates the practice of the present invention for the determination of *Streptococcus A* antigen. It also compares the practice of the present invention using a wash solution having a salt concentration of at least 0.25 molar (that is, at least 0.25 ionic strength) to an assay outside the scope of this invention using a wash solution containing less than 0.25 molar salt (that is, less than 0.25 ionic strength).

Core-shell polymeric latex particles containing a red dye (Oil Red EGN) in the core were prepared by imbibing the dye into the particles that had been prepared using core/shell polymerization techniques. The core of the particles was composed of poly(styrene-co-2-acetoacetoxyethyl methacrylate) (70:30 weight ratio) while the shell was composed of poly (m,p-chloromethylstyrene). The average diameter of the particles was about 0.45 micrometer. Monoclonal antibodies to *Streptococcus A* antigen were immobilized on these particles as follows: to 0.6 ml of 50 mmolar borate buffer (pH 8.5) was added 0.1 mg of total protein comprised of a 10:1 mixture of anti-Strep A antibody (purchased as a 2.9 mg/ml solution in phosphate buffered saline solution, known in the art as PBS) and casein (10 mg/ml water). After mixing, 41.5  $\mu$ l of a 5% suspension of the polymeric latex particles were added (to provide 0.3% solids) and the resulting solution was rotated (end-over-end) for 24 hours at 37 °C to effect covalent attachment of the antibody to the outer surfaces of the particles and the formation of an agglutination indicator reagent.

A solution of succinic anhydride (10 mg/ml dimethyl sulfoxide) was added to a suspension of the agglutination indicator reagent described above at a weight ratio of 1 part anhydride to 1 part total protein. The resulting suspension was mixed for four hours at 25 °C, centrifuged for 5 minutes at 7000 rpm and the resulting pellet was resuspended in 0.1 molar glycine buffer (pH 8.5) to a concentration of 0.3% solids.

An isolate of *Streptococcus A* obtained from a local hospital was used in the assays of this example. *Streptococcus A* antigen was extracted from an isolate at 25 °C for 1 minute using a solution of equal volumes of sodium nitrite (8 molar) and citric acid (0.2 molar). The solution was then neutralized with an equal volume of

3-(N-morpholino)propanesulfonic acid buffer (2 molar, pH 7.5) containing ethylenediaminetetraacetic acid (75 mmolar).

A nylon 66 microporous membrane (5  $\mu\text{m}$  average pore size) was incorporated into a test well of a disposable test device and pretreated by washing with 100  $\mu\text{l}$  of a 2% succinylated casein solution.

5 A mixture of sodium chloride (80  $\mu\text{l}$ , 1 molar), the agglutination indicator reagent suspension described above (40  $\mu\text{l}$ ), and extracted antigen (80  $\mu\text{l}$ ) containing about  $4.2 \times 10^5$  colony-forming units per ml was added to the test well of the test device containing the membrane, and incubated therein for two minutes at 25°C. The fluid was then allowed to drain into a compartment below the membrane, and the agglutinate on the membrane was washed with 150  $\mu\text{l}$  of the wash fluids shown in Table I below. Control A was distilled  
10 water while Control B was a wash solution containing 0.025 molar sodium chloride (that is, 0.025 ionic strength).

After the washing step, the amount of dye in the agglutinate on the membrane was measured at 540 nm using reflectance measuring equipment. The Williams-Clapper transform (J. Optical Soc. Am., 43, p 595, 1953) was used to calculate transmission density values. The results of the measurements are shown in  
15 Table I below as  $\Delta D_T$ , the difference between the dye formed from the test samples and background readings from controls containing no antigen. It can be seen from the data that the assays of the present invention using a high concentration of salt in the wash solution provided a readily observable agglutinate on the membrane. The Control A and B assays, however, in which little dye was observed, demonstrate that the lack of sufficient salt in the wash solution prohibits adequate retention of agglutinate on the membrane.

20

T A B L E I

	<u>Ionic Strength</u>	<u><math>\Delta D_T</math></u>
25	0 (Control A)	0.018
	0.025 (Control B)	0.022
	0.25	0.148
	0.5	0.152
30	1.0	0.170
	3.0	0.114

35

#### Example 2: Determination of Gonorrhea

This example demonstrates the practice of the present invention for the determination of gonorrhea. The agglutination indicator reagent used in this example was composed of latex particles comprised of poly-  
40 (styrene-co-m,p-chloromethylstyrene-co-2-hydroxyethyl acrylate) (76:23:1 weight ratio) into which had been imbibed 5%, by weight, of europium (III) (thenoyltrifluoroacetone)<sub>3</sub> along with trioctylphosphine oxide in the ratio of 1 part chelate to 2 parts oxide according to the procedures described in Belgian Patent 843,647. The particles had an average diameter of about 0.45 micrometer.

Monoclonal antibodies to the PI antigen of the serogroup B of *Neisseria gonorrhea* (also known in the art as the PIB antigen) were immobilized on the particles described above as follows: to 1.3 ml of 50 mmolar borate buffer (pH 8.5) was added 0.15 ml of 1.08 mg/ml antibody solution in phosphate buffered saline (PBS) and 0.32 ml of a 1 mg/ml solution of casein in water. After mixing, 41.5  $\mu\text{l}$  of a 5% suspension of the latex particles described above were added, and the resulting solution was mixed at 37°C for 24 hours. Succinic anhydride (0.174 ml of 10 mg/ml dimethyl sulfoxide solution) was added, and the resulting  
50 solution was mixed at 22°C for four hours. This solution was then centrifuged for 10 minutes and the resulting pellet was resuspended in 0.1 molar glycine (pH 8.5) to give a mixture containing 0.3% solids of agglutination indicator reagent.

The PIB antigen was extracted from a specimen of *Neisseria gonorrhea* using a mixture of 1% ethanolamine and 10 mmolar ethylenediaminetetraacetic acid, followed by sonication and filtration.

55 A nylon 66 microporous membrane having an average pore size of 5 micrometers was pretreated by dipping it into a 2% casein solution. A mixture of sodium chloride (50  $\mu\text{l}$ , 6 molar), antigen solution (50  $\mu\text{l}$ ) having a specific amount of antigen (nanogram) and the agglutination indicator solution described above (50  $\mu\text{l}$ ) was added to a test tube, incubated at 22°C for 30 minutes, then filtered through the treated

microporous membrane. The resulting agglutinate on the membrane was washed with 0.15  $\mu$ l of 1 molar tricine (pH 8.6). The amount of agglutinate was determined by measuring the amount of fluorescence in the agglutinate using standard surface fluorescence measuring equipment (excitation, 342 nm and emission, 610 nm). A Control solution containing specific amounts of an extract of a different antigen (that is, the PI antigen of the serogroup A of *Neisseria gonorrhea*, or also known as the PIA antigen) was treated in the same manner in order to measure nonspecific interactions with the antibodies to the PIB antigen. Table II below shows the results of these tests. It is clear that the assay of this invention can be used to determine a desired antigen of a specific serogroup of gonorrhea.

T A B L E II

PIB Antigen Concentration (ng)	Relative Fluorescence	
	Test	Control
100	107	32
10	332	120
1	248	73

#### Example 3: Assay for Human Chorionic Gonadotropin

This example demonstrates the practice of the present invention for the determination of human chorionic gonadotropin (hCG).

Core/shell polymeric particles were imbibed with Oil Red EGN dye according to known procedures. The particle cores were composed of poly(styrene-co-2-acetoacetoxyethyl methacrylate) (85:15 weight ratio), and the particle shells were composed of poly(m,p-chloromethylstyrene-co-methacrylic acid) (99.8:0.2 weight ratio). The particles had an average diameter of about 0.32 micrometer.

Monoclonal antibodies to two different epitopic sites of hCG were immobilized on these particles as follows: to 0.6 ml of 50 mmolar borate buffer (pH 8.5) were added 0.1 mg of 10:1 mixture of hCG antibody (2.9 mg/ml phosphate buffered saline solution) and casein (10 mg/ml water). After mixing, 41.5  $\mu$ l of a 5% suspension of the latex particles described above were added and the resulting suspension was rotated (end-over-end) for 24 hours at 37°C to effect covalent attachment of the antibodies to the particles and formation of an agglutination indicator reagent.

A solution of succinic anhydride (10 mg/ml dimethyl sulfoxide) was added to a mixture of the agglutination indicator reagent at a weight ratio of 1 part anhydride to 1 part total protein, and the resulting mixture was mixed for 4 hours at 25°C, centrifuged for 5 minutes at 7000 rpm. The resulting pellet was resuspended in 0.1 molar glycine (pH 8.5) to a concentration of 0.3% solids.

Various amounts of hCG (milli I.U./ml) were added to phosphate buffered saline solutions (0.1 molar sodium phosphate and 0.15 molar sodium chloride) containing 0.5% bovine serum albumin. A nylon 66 microporous membrane having an average pore size of about 5 micrometers was incorporated into a test well of a disposable test device similar to that described in Example 1 above. This membrane was washed with 2 drops of a 1% aqueous solution of succinylated casein. The hCG concentration in milli I.U. is defined as 5000 milli I.U. being equivalent to 1 microgram of purified hCG.

A mixture of 60  $\mu$ l of 4 molar sodium chloride, 1 molar tricine buffer (pH 8.6), 60  $\mu$ l of suspension of the agglutination indicator reagent described above and 240  $\mu$ l of the hCG solutions described above was added to test tubes, gently mixed and allowed to incubate at 25°C for 10 minutes. A portion of each solution (300  $\mu$ l) was added to the test well containing the membrane and allowed to flow through the membrane. Agglutinate formed on the membrane did not flow through, however. It was washed with 300  $\mu$ l of a 1 molar sodium chloride solution, and the amount of dye in the agglutinate was measured at 540 nm as described in Example 1. The results of these measurements are shown in Table III below as transmission density ( $D_T$ ). It indicates that the assay of this invention can be used to determine hCG.



T A B L E    I I I

<u>hCG Antigen (milli I.U./ml)</u>	<u>D<sub>T</sub></u>
0	0.043
500	0.047
1000	0.133

## Claims

1. An agglutination method for the determination of a multivalent immune species comprising:
  - (a) contacting an aqueous liquid containing the species in free form with a reagent comprising water-insoluble particles having tracer molecules associated therewith and receptor molecules reactive with the species bound to the surface thereof, so as to form an agglutinate of the reaction product of the species and the receptor molecules,
  - (b) simultaneously or subsequent to contacting step (a), contacting the agglutinate with a microporous water-insoluble membrane having an average pore size which is at least 5 times greater than the average diameter of the water-insoluble particles,
  - (c) washing unagglutinated residual materials through the membrane while leaving the agglutinate thereon with a wash solution, and
  - (d) determining the amount of tracer either in the agglutinate remaining on the membrane or the residual materials,
 the method characterized wherein the wash solution has a pH of from 5 to 10 and an ionic strength of at least 0.25.
2. The method as claimed in claim 1 wherein the wash solution has an ionic strength of from 0.5 to 3.
3. The method as claimed in either of claims 1 or 2 wherein the wash solution has a pH of from 6 to 9.
4. An agglutination method for the determination of Streptococcus A in a biological sample comprising:
  - (a) extracting Streptococcus A antigen from the sample with an extraction composition,
  - (b) contacting the extracted antigen with an agglutination reagent comprising water-insoluble, polymeric latex particles having tracer molecules distributed therein and antibodies reactive with the antigen covalently bound to the surface thereof, so as to form an agglutinate of the reaction product of the antigen and the antibodies, the contacting being carried out in an aqueous mixture in the presence of a microporous water-insoluble membrane having an average pore size which is at least five times greater than the average diameter of the water-insoluble particles,
  - (c) washing unagglutinated residual materials through the membrane while leaving the agglutinate thereon with a wash solution, and
  - (d) determining the amount of tracer in the agglutinate remaining on the membrane,
 the method characterized wherein the wash solution has a pH of from 6 to 9 and an ionic strength from 0.25 to 3.
5. The method as claimed in claim 4 wherein the wash solution has an ionic strength of from 0.5 to 3.
6. The method as claimed in either of claims 4 or 5 wherein the wash solution comprises sodium chloride.
7. The method as claimed in any of claims 1 to 6 carried out using a disposable test device containing the microporous membrane.
8. A method for detecting Streptococcus A comprising:
  - (i) providing an applicator including an applicator stick and a fibrous swab and collecting a biological sample with the swab,
  - (ii) providing an extraction composition comprising sodium nitrite and citric acid for effecting release of Streptococcus A antigen from the swab, dipping the swab in the extraction composition and

incubating the swab within the extraction composition for a period of time sufficient to release antigen from the swab,

(iii) neutralizing the solution of the extracted antigen,

(iv) contacting the neutralized solution of extracted antigen with an agglutination indicator reagent comprising water-insoluble, polymeric core-shell latex particles having an average diameter of from 0.1 to 0.7 micrometer, and having molecules of a colorimetric dye distributed substantially in the particle cores and comprising antibodies reactive with the antigen bound to the surface thereof, so as to form an agglutinate of the reaction product of the antigen and the antibodies, the contacting being carried out in the presence of a microporous water-insoluble membrane mounted in a disposable test device, the membrane having an average pore size which is at least 5 times greater than the average diameter of the water-insoluble particles,

(v) washing unagglutinated residual materials through the membrane while leaving the agglutinate thereon with a wash solution, and

(vi) determining the amount of colorimetric dye in the agglutinate remaining on the membrane,

the method characterized wherein the wash solution has a pH of from 6 to 9 and an ionic strength of from 0.5 to 3.

9. The method as claimed in claim 8 wherein the wash solution comprises sodium chloride.

10. A test kit for the determination of a multivalent immune species comprising:

an agglutination indicator reagent comprising water-insoluble particles having tracer molecules associated therewith and receptor molecules reactive with the species bound to the surface thereof, and

a wash solution,

the kit characterized wherein the wash solution has a pH of from 5 to 10 and an ionic strength of at least 0.25.

## Patentansprüche

1. Agglutinationsmethode für die Bestimmung einer mehrwertigen Immunspezies, bei der man:

(a) eine wäßrige Flüssigkeit, welche die Spezies in freier Form enthält, mit einem Reagens in Kontakt bringt, das aufweist in Wasser unlösliche Partikel mit Tracer-Molekülen, die mit den in Wasser unlöslichen Partikeln assoziiert sind, sowie mit Rezeptor-Molekülen, die mit der Spezies zu reagieren vermögen und an die Oberfläche gebunden sind, zum Zwecke der Bildung eines Agglutinates aus dem Reaktionsprodukt der Spezies und den Rezeptor-Molekülen,

(b) das Agglutinat gleichzeitig oder nach der Kontaktierungsstufe (a) mit einer mikroporösen in Wasser unlöslichen Membran in Kontakt bringt, die eine durchschnittliche Porengröße aufweist, die mindestens 5 mal größer ist als der durchschnittliche Durchmesser der in Wasser unlöslichen Partikel,

(c) Auswaschen des nicht agglutinierten restlichen Materials durch die Membran unter Zurücklassen des Agglutinates mittels einer Waschlösung und

(d) Bestimmung der Menge an Tracer entweder in dem auf der Membran zurückgebliebenen Agglutinat oder den restlichen Materialien,

dadurch gekennzeichnet, daß die Waschlösung einen pH-Wert von 5 bis 10 aufweist sowie eine Ionenstärke von mindestens 0,25.

2. Verfahren nach Anspruch 1, bei dem die Waschlösung eine Ionenstärke von 0,5 bis 3 aufweist.

3. Verfahren nach einem der Ansprüche 1 oder 2, bei dem die Waschlösung einen pH-Wert von 6 bis 9 aufweist.

4. Agglutinationsmethode zur Bestimmung von Streptokokkus A in einer biologischen Probe, bei der man

(a) Streptokokkus-A-Antigen aus der Probe mit einer Extraktions-Zusammensetzung extrahiert,

(b) das extrahierte Antigen mit einem Agglutinationsreagens in Kontakt bringt, das aufweist in Wasser unlösliche polymere Latexteilchen mit Tracer-Molekülen, die hierin verteilt sind sowie Antikörpern, die mit dem Antigen zu reagieren vermögen und kovalent an die Oberfläche der Teilchen gebunden sind, zum Zwecke der Bildung eines Agglutinates aus dem Reaktionsprodukt des Antigens und den Antikörpern, wobei das Inkontaktbringen in einer wäßrigen Mischung in

- Gegenwart einer mikroporösen, in Wasser unlöslichen Membran erfolgt, die eine durchschnittliche Porengröße aufweist, die mindestens 5 mal größer ist als der durchschnittliche Durchmesser der in Wasser unlöslichen Partikel,
- (c) Auswaschen der restlichen nichtagglutinierten Materialien durch die Membran unter Zurücklassen des Agglutinates auf der Membran unter Verwendung einer Waschlösung und
- (d) Bestimmung der Menge an Tracer in dem Agglutinat, das auf der Membran zurückbleibt, dadurch gekennzeichnet, daß die Waschlösung einen pH-Wert von 6 bis 9 sowie eine Ionenstärke von 0,25 bis 3 aufweist.
5. Verfahren nach Anspruch 4, bei dem die Waschlösung eine Ionenstärke von 0,5 bis 3 aufweist.
6. Verfahren nach einem der Ansprüche 4 oder 5, bei dem die Waschlösung Natriumchlorid enthält.
7. Verfahren nach einem der Ansprüche 1 bis 6, das unter Verwendung eines Wegwerf-Testgerätes mit der mikroporösen Membran durchgeführt wird.
8. Verfahren zur Bestimmung von Streptokokkus A mit:
- (i) Bereitstellen eines Applikators mit einem Applikatorstab und einem fasrigen Bausch und Aufnehmen einer biologischen Probe mit dem Bausch,
- (ii) Bereitstellen einer Extraktions-Zusammensetzung mit Natriumnitrit und Zitronensäure für die Bewirkung der Freisetzung von Streptokokkus-A-Antigen aus dem Bausch, Eintauchen des Bausches in die Extraktions-Zusammensetzung und Inkubieren des Bausches innerhalb der Extraktions-Zusammensetzung eine solche Zeit lang, die ausreicht, um Antigen aus dem Bausch freizusetzen.
- (iii) Neutralisieren der Lösung des extrahierten Antigens,
- (iv) Kontaktieren der neutralisierten Lösung des extrahierten Antigens mit einem Agglutinations-Indikator-Reagens mit in Wasser unlöslichen polymeren Kern-Hüllen-Latexpartikeln mit einem mittleren Durchmesser von 0,1 bis 0,7 Mikrometern, wobei Moleküle eines colorimetrischen Farbstoffes im wesentlichen in den Partikel- oder Teilchenkernen verteilt sind sowie mit Antikörpern, die mit dem Antigen zu reagieren vermögen und an die Oberfläche gebunden sind, zum Zwecke der Bildung eines Agglutinates des Reaktionsproduktes des Antigens mit den Antikörpern, wobei das Kontaktieren in Gegenwart einer mikroporösen, in Wasser unlöslichen Membran erfolgt, die in einem Wegwerf-Testgerät befestigt ist, wobei die Membran eine durchschnittliche Porengröße aufweist, die um mindestens 5 mal größer ist als der mittlere Durchmesser der in Wasser unlöslichen Teilchen oder Partikel,
- (v) Auswaschen der restlichen nichtagglutinierten Materialien durch die Membran unter Zurücklassen des Agglutinates auf der Membran mittels einer Waschlösung und
- (vi) Bestimmung der Menge des colorimetrischen Farbstoffes in dem auf der Membran zurückgebliebenen Agglutinat, dadurch gekennzeichnet, daß die Waschlösung einen pH-Wert von 6 bis 9 und eine Ionenstärke von 0,5 bis 3 aufweist.
9. Verfahren nach Anspruch 8, bei dem die Waschlösung Natriumchlorid enthält.
10. Test-Kit für die Bestimmung einer mehrwertigen Immunspezies mit:
- einem Agglutinations-Indikator-Reagens mit in Wasser unlöslichen Teilchen mit Tracer-Molekülen, die mit den Teilchen assoziiert sind und Rezeptor-Molekülen, die mit der Spezies zu reagieren vermögen und an die Oberfläche gebunden sind und einer Waschlösung,
- wobei der Kit dadurch gekennzeichnet ist, daß die Waschlösung einen pH-Wert von 5 bis 10 und eine Ionenstärke von mindestens 0,25 aufweist.

## Revendications

1. Procédé d'agglutination pour le dosage d'une espèce immunologique multivalente qui consiste à :
- (a) mettre en contact un liquide aqueux contenant l'espèce sous forme libre avec un réactif comprenant des particules insolubles dans l'eau associées à un traceur et des molécules réceptrices liées à la surface des particules qui réagissent avec l'espèce, afin de former un agglutinat qui est le produit de réaction de l'espèce avec des molécules réceptrices,

(b) de façon simultanée ou non à l'étape (a), mettre en contact l'agglutinat avec une membrane insoluble dans l'eau microporeuse ayant une taille de pores moyenne qui est au moins 5 fois supérieure au diamètre moyen des particules insolubles dans l'eau,

(c) laver avec une solution de lavage pour que les résidus non agglutinés traversent la membrane tout en laissant l'agglutinat sur la membrane, et

(d) déterminer la quantité de traceur soit dans l'agglutinat restant sur la membrane, soit dans les résidus,

le procédé étant caractérisé en ce que la solution de lavage a un pH compris entre 5 et 10 et une force ionique d'au moins 0,25.

2. Procédé selon la revendication 1 dans lequel la solution de lavage a une force ionique comprise entre 0,5 et 3.

3. Procédé selon l'une quelconque des revendications 1 ou 2 dans lequel la solution de lavage a un pH compris entre 6 et 9.

4. Procédé d'agglutination pour le dosage du Streptocoque A dans un échantillon biologique qui consiste à :

(a) extraire l'antigène Streptocoque A d'un échantillon avec une composition d'extraction,

(b) mettre en contact l'antigène extrait avec un réactif d'agglutination comprenant des particules de latex dans lesquelles sont réparties des traceurs et des anticorps liés de façon covalente à la surface des particules qui réagissent avec l'antigène, afin de former un agglutinat qui est le produit de réaction de l'antigène avec les anticorps, la mise en contact s'effectuant dans un mélange aqueux en présence d'une membrane insoluble dans l'eau microporeuse ayant une taille de pores moyenne qui est au moins 5 fois supérieure au diamètre moyen des particules insolubles dans l'eau,

(c) laver avec une solution de lavage pour que les résidus non agglutinés traversent la membrane tout en laissant l'agglutinat sur la membrane, et

(d) déterminer la quantité de traceur dans l'agglutinat restant sur la membrane,

le procédé étant caractérisé en ce que la solution de lavage a un pH compris entre 6 et 9 et une force ionique comprise entre 0,25 et 3.

5. Procédé selon la revendication 4 dans lequel la solution de lavage a une force ionique comprise entre 0,5 et 3.

6. Procédé selon l'une quelconque des revendications 4 ou 5 dans lequel la solution de lavage comprend du chlorure de sodium.

7. Procédé selon l'une quelconque des revendications 1 à 6 mise en oeuvre en utilisant un dispositif de test à usage unique contenant la membrane microporeuse.

8. Procédé pour le dosage du Streptocoque A qui consiste à :

(i) utiliser un applicateur comprenant un bâton applicateur et un tampon fibreux pour collecter un échantillon biologique avec le tampon,

(ii) constituer une composition d'extraction comprenant du nitrite de sodium et de l'acide citrique pour extraire l'antigène Streptocoque A du tampon, plonger le tampon dans la composition d'extraction et incuber la composition d'extraction contenant le tampon pendant un période suffisamment longue pour extraire l'antigène du tampon,

(iii) neutraliser la solution de l'antigène extrait,

(iv) mettre en contact la solution neutralisée de l'antigène extrait avec un réactif indicateur de l'agglutination comprenant des particules de latex à coeur et à coque insolubles dans l'eau ayant un diamètre moyen compris entre 0,1 et 0,9  $\mu\text{m}$ , et ayant des molécules d'un colorant pour dosage colorimétrique réparties dans le coeur des particules et des anticorps liés de façon covalente à la surface des particules qui réagissent avec l'antigène, afin de former un agglutinat qui est le produit de réaction de l'antigène avec les anticorps, la mise en contact s'effectuant en présence d'une membrane insoluble dans l'eau microporeuse placée dans un dispositif de test à usage unique, la membrane ayant une taille de pores moyenne qui est au moins 5 fois supérieure au diamètre moyen des particules insolubles dans l'eau,

(v) laver avec une solution de lavage pour que les résidus non agglutinés passent au travers de la membrane et que l'agglutinat reste sur la membrane, et

(vi) déterminer la quantité de colorant pour dosage colorimétrique dans l'agglutinat restant sur la membrane,

5 le procédé étant caractérisé en ce que la solution de lavage a un pH compris entre 6 et 9 et une force ionique comprise entre 0,25 et 3.

9. Procédé selon la revendication 8 dans lequel la solution de lavage comprend du chlorure de sodium.

10 10. Nécessaire de test pour le dosage d'une espèce immunologique multivalente qui comprend :

un réactif indicateur de l'agglutination comprenant des particules insolubles dans l'eau associées à un traceur et des molécules réceptrices liées à la surface des particules, qui réagissent avec l'espèce, et

une solution de lavage,

15 le nécessaire étant caractérisé en ce que la solution de lavage a un pH compris entre 5 et 10 et une force ionique d'au moins 0,25.

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